TRANSFERRIN-IRON-ANION COMPLEX TRANSPORT INTO HeLa CELLS

Réka SINKLER[1], Melinda VERÉBI-KERTÉSZ[2], Dóra NÉMETH[3], Marianna PAP[4], Ferenc KILÁR[5]

Metals can be transported into cells via the central protein, transferrin, which is a major component of the blood serum. Iron uptake of human cells occurs through the serum transferrin – transferrin receptor pathway, providing also the possibility for site-specific delivery of various therapeutic metal ions, drugs and several small molecules. The aim of this study was to investigate the uptake of fluorescently labeled transferrin-iron-anion complexes and to follow the effect on the proliferation of immortal HeLa cell line. The results represent a solid starting point for targeted drug therapy methods, especially with the possible application of anionic drug substances.

Keywords: transferrin complexes, FITC labeling, HeLa cells

1. Introduction

Members of the transferrin family (ovotransferrin, lactoferrin, melanotransferrin and human serum transferrin) play important role in the iron storage and transport [1-3]. Melanotransferrin, having only one lobe (N-lobe), and consequently only one active iron binding site, is not involved in the iron metabolism, but it plays an important role in the proliferation and tumorigenesis of melanoma cells [4]. Serum transferrin (sTf) is the most important member of the transferrin family. It is synthesized in the liver and secreted into the blood circulation [5]. The main role of sTf, having two – the N and the C-terminal – lobes with respective iron binding sites, is the transport of iron to the place of utilization, primarily to hemoglobin synthesis. Besides this function it has other important functions, like the protection of the organism against microbial infections, by binding iron(III) ions causing iron deficiency in the pathogenic

[1] PhD student., Dept. of Analytical Chemistry and Environmental Engineering, University POLITEHNICA of Bucharest, Romania, e-mail: sinklerreka@uni.sapientia.ro
[2] Biologist, 2nd Dept. of Medicine and Nephrological Sciences, Medical School, University of Pécs, Hungary, e-mail: melinda.kertesz@aok.pte.hu
[3] MSc student, Institute of Bioanalysis, Medical School, University of Pécs, Hungary, e-mail: nemeth.doraa95@gmail.com
[4] Associate professor, Dept. of Medical Biology, Medical School, University of Pécs, Hungary, e-mail: marianna.pap@aok.pte.hu
[5] Professor, Institute of Bioanalysis, Medical School and Szentágothai Research Centre, University of Pécs, Hungary, e-mail: ferenc.kilar@aok.pte.hu

microorganisms [6, 7]. The concentration of serum transferrin is around 2.5 mg/mL in human blood, but only 30% of the iron binding sites is saturated with iron [8, 9]. Although, transferrin's main role is binding of iron(III), it can bind other cations can, as well [10]. The metal-ion binding occurs only in the presence of a synergistic anion (in each lobe), which occupies two ligand positions in the iron(III) coordination area. *In vivo*, bicarbonate anions take place in the iron binding process, but *in vitro* other anions, *e.g.* oxalate, can replace the bicarbonate [11]. The fact that other ions can contribute to the iron binding gives a basis for targeted drug transport into cells via transferrin uptake.

Cancer treatment need adequate multivariable dose-restricting factors for the success of therapy, to increase quality of life, and to ensure complete long-term remission rates [12]. Targeted drug therapy can be a solution by enhancing cellular uptake and decreasing systemic toxicity. Transferrin complexes can be used as options to targeted drug delivery via the binding to transferrin receptors (TfR). The upregulation of TfR expression on metastatic and drug resistant tumors shows selectivity to cancer cells, which provides the elevated uptake of transferrin [13-16].

Oxalate has concentration-dependent toxic effects. Oxalate induced cell death was determined mainly in renal epithelial cells. Studies using HK-2, LLCPK1, MDCK cells indicate that increased concentration of oxalate has cytotoxic effects mediated by cellular apoptosis, because oxalate exposure induces changes in membrane integrity, in the release of cellular enzymes, and in membrane lipid peroxidation [17-21].

The aim of this study was to follow the incorporation of transferrin-iron-anion complexes labeled with amino-reactive fluorescent isothiocyanate (FITC) into HeLa cells, and to monitor the effect of the intracellular transport of bioactive anionic compounds on the cells.

2. Materials and Methods

2.1. Chemicals

Lyophilized human serum transferrin was obtained in iron-free form (Lee Biosolutions, St.Louis, USA) and in iron-saturated form, *i.e.* as diferric-bicarbonate-transferrin complex (Sigma-Aldrich, Seelze, Germany). Fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich (Seelze, Germany), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, sodium-hydrogen carbonate and oxalic acid were obtained from Reanal (Budapest, Hungary).

2.2. Preparation and fluorescent labeling of transferrin-complexes

Stock solutions of transferrin-iron(III)-anion complexes were prepared from iron-free transferrin as it has been described previously [22]. The

bicarbonate-containing diferric-transferrin sample was labeled as $Fe_2Tf(HCO_3)_2$-P and the oxalate-containing diferric-transferrin was labeled as $Fe_2Tf(ox)_2$-P. A stock solution of the commercially available diferric-bicarbonate transferrin was labeled as $Fe_2Tf(HCO_3)_2$-C. Fluorescence labeling of the transferrin solutions was obtained by using 2 mM stock solution of FITC diluted in 100 mM $NaHCO_3$ buffer, pH=8.75. The labeling was set to provide FITC:transferrin conjugates with 1:1 molar ratio as described earlier [23]. The complete reaction was obtained by incubation in dark for 24 hours at room temperature. The excess of FITC was removed by dialyzis in 20 mM $NaHCO_3$ buffer, pH =7, for 24 hours.

2.3. Cell culture conditions

HeLa (ATCC-CCL-2, human cervical cancer) cells were a kind gift of Prof. G. M. Cooper (Boston University, Boston, MA, USA). Cells were cultured at 37ºC in an atmosphere containing 5% CO_2, in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% Fetal bovine serum (FBS) (PAN Biotech, Aidenbach, Germany).

2.4. Cell treatment with FITC-labeled transferrin complexes

HeLa cells were plated on 24-well cell culture plates, on poly-L-lysine coated coverslips (10^4 cells/well). 24 hours later, the culture medium was replaced by medium containing 50 µg/mL FITC-labeled iron-free-Tf, $Fe_2Tf(HCO_3)_2$-C, $Fe_2Tf(HCO_3)_2$-P, $Fe_2Tf(ox)_2$-P samples or containing 50 µg/mL unlabeled iron-free-Tf and $Fe_2Tf(HCO_3)_2$-C samples. All solutions were filter-sterilized (Millex Syringe-driven filter unit) before use. The cells were incubated with the appropriate Tf-complexes for 10 minutes or for 1 hour, respectively. After incubation, the cells were fixed with 500 µl 4% paraformaldehyde (PFA) at room temperature for 30 minutes. The cells were stained by Hoechst 33342 dye (Calbiochem, Darmstadt, Germany), which was dissolved (1:2000) in phosphate buffered saline (PBS). After staining the excess dye was removed by washing in PBS 3 times for 5 minutes. After the washing steps, the coverslips were removed from the wells and placed onto glass slides and embedded with 3 µl Vectashield (Vector Laboratories, Burlingame, CA, USA) mounting medium. For visualization, an Olympus FluoView 1000 confocal laser scanning fluorescence microscope (Olympus, Center Valley, PA, USA) was used. The FITC was excited in photon counting and sequential mode creating single-plane images. The experiment was performed twice, each time in duplicates.

2.5. Proliferation assay

2×10^3 HeLa cells/well were plated on 24-well plates. The next day the cells were treated with transferrin-containing media, *i.e.*, containing 0.2 g/L iron-free-Tf or $Fe_2Tf(HCO_3)_2$-C or $Fe_2Tf(HCO_3)_2$-P or $Fe_2Tf(ox)_2$-P complexes. The cell proliferation experiments lasted 3 or 6 days, respectively. The cells, which were cultured for 6 days were treated with fresh culture medium with the same

transferrin content after three days. At the end of the proliferation experiments the culture medium was removed, and the cells were trypsinized with 500 µl trypsin/EDTA solution to release the cells from the surface of the wells. The reaction was stopped with 500 µl culture medium. The cells were centrifuged and resuspended in culture medium, then counted in a Bürker chamber. The proliferation assay was performed in triplicates.

3. Results

Transferrin-complexes containing iron(III) ions and bicarbonate or oxalate anions were prepared. The internalization of these complexes into HeLa cells was verified using confocal laser scanning fluorescent microscopy.

3.1. Internalization of FITC-labeled complexes into HeLa cells

Cells were incubated with different FITC-labeled transferrin complexes in order to assess internalization capacity and to visualize their intracellular localization. Since the cells contain molecules (*e.g.* reduced pyridine nucleotides - NADH, oxidized flavins - FMN, FAD, which can emit fluorescent light), this intrinsic property of cells, causing the so-called auto fluorescence, should be considered in the experiments. Therefore, the green fluorescence of the control cells was set as low as possible and the same laser settings was used to analyze the cells treated with FITC-labeled transferrin-complexes.

Representative results of non-treated HeLa cells and cells treated with FITC-labeled diferric-bicarbonate-transferrin complexes are shown in Fig. 1.

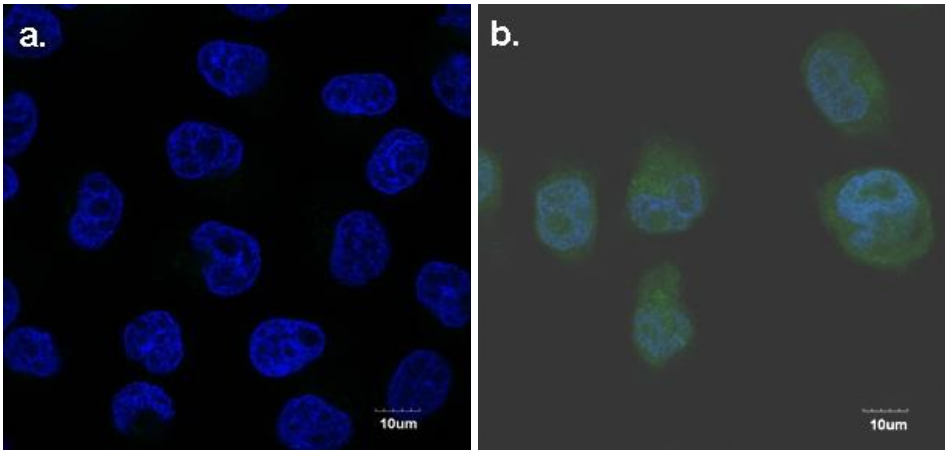

Fig. 1. Confocal laser scanning fluorescence microscopy images of HeLa cells: (a) control (non-treated) HeLa cells, (b) HeLa cells 10 minutes after treatment with FITC-labeled diferric-bicarbonate-transferrin,

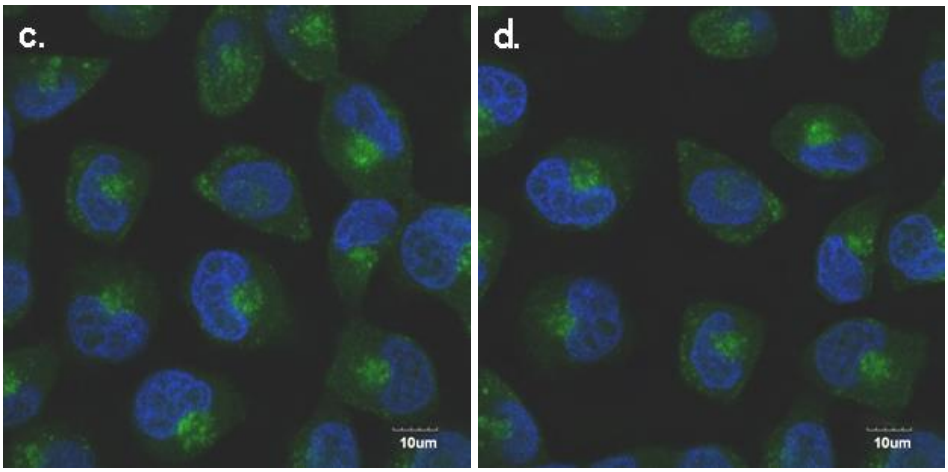

Fig. 1. Confocal laser scanning fluorescence microscopy images of HeLa cells: (c) HeLa cells 1 hour after treatment with FITC-labeled diferric-bicarbonate-transferrin, and (d) HeLa cells 1 hour after treatment with FITC-labeled oxalate-containing diferric-transferrin. The blue color indicates the cell nuclei stained by Hoechst 33342 dye, and the green staining shows the distribution of FITC-labeled transferrin.

In order to see the cell nuclei staining with Hoechst 33342 dye was performed appearing as blue color on the pictures. The 10 minutes incubation with FITC-labeled transferrin-complexes (green color) could be seen on the membrane of cells as bright spots and scattered inside the cells, as well, showing that FITC-labeled complexes are already internalized into the cells appearing as a homogenous staining (Fig. 1b). 1 hour after the treatment the FITC-labeled transferrin-complexes are accumulated in vesicles next to the nucleus (Figs. 1c and 1d). The treatment of the cells with all kinds of transferrin complexes were performed in duplicates and showed FITC accumulation in the cells in all cases.

3.2 Cell proliferation monitoring

After proving the internalization of transferrin-complexes into the HeLa cells cell-proliferation-studies were performed.

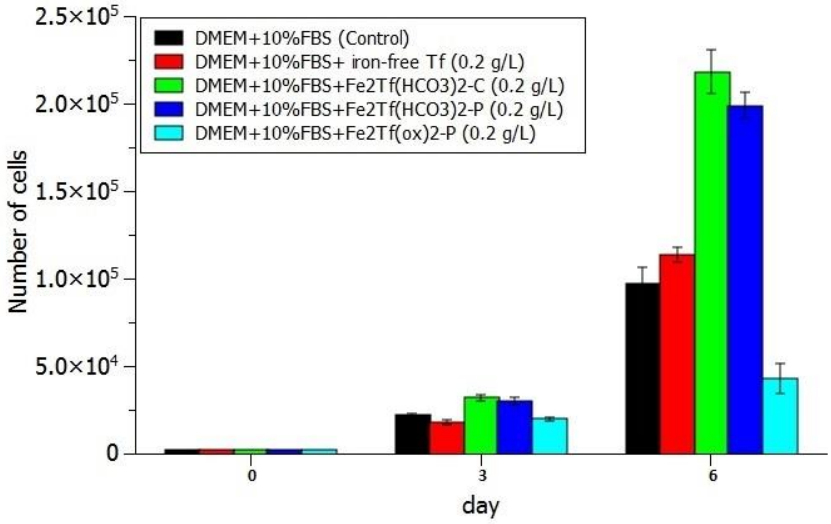

Fig. 2. Averages of cell numbers in proliferation experiments. HeLa cells (2×10^3 cells) were cultured in medium containing 10 % FBS. The control experiment was performed only in the medium, while culturing was followed in the presence of added iron-free transferrin or various transferrin-iron-anion complexes, as well. The experiments lasted for 3 or 6 days.

HeLa cells were plated (2×10^3 cells/well) on cell culture plates and cultured in medium containing added human transferrin samples, such as iron-free Tf, $Fe_2Tf(HCO_3)_2$-C, $Fe_2Tf(HCO_3)_2$-P or $Fe_2Tf(ox)_2$-P. The concentration of the protein in the medium was 0.2 g/L. The cells were grown for 3 or 6 days and counted after finishing the experiments. Cells in the presence of added human diferric-bicarbonate-transferrin complex proliferated *ca.* twice as much as the control cells. Both, the commercially available diferric-transferrin ($Fe_2Tf(HCO_3)_2$-C) and the diferri-transferrin prepared by us ($Fe_2Tf(HCO_3)_2$-P) had the same proliferation effect.

Interestingly, the presence of the iron-free transferrin in the culture medium also caused a slight increase in the proliferation. However, the replacement of the bicarbonate with oxalate (*i.e.*, applying the $Fe_2Tf(ox)_2$-P complex) caused a strong inhibition in cell-proliferation as it was observed after 6 days.

4. Conclusions

HeLa cells were selected for the studies, since it is well known, that the HeLa cells express TfRs [24], thus this cell line constitutes a valid model for internalization, as well as, cell fitness and cell growth properties. The internalization and intracellular trafficking of the Tf-complexes was monitored by using a confocal laser scanning fluorescence microscope, which visualized the

proteins labeled with the fluorescent FITC dye. The biological effect of human serum transferrin-complexes containing different anions on the proliferation of HeLa cells was also studied. It was found that the synergistic oxalate anion bound at the iron-binding site is responsible for a significantly lower proliferation rate of HeLa cells, while the addition of transferrin complexes containing bicarbonate increased the proliferation compared to the control cells.

This observation directs our interest towards the role and possible "bio-application" of anionic substances or drugs. The overall results prove that choosing appropriate anions in Tf-complexes may take part in drug delivery via receptor mediated endocytosis. The transferrin complexes show the possibility to deliver anionic molecules into tumor cells, which can be applied in targeted anticancer therapy.

Acknowledgements

This work was supported by the NKFIH K-125275 and the PTE-AOK-KA-2017-19 grants.

R E F E R E N C E S

[1] *F. Giansanti, L. Leboffe, G. Pitari et al.*, "Physiological roles of ovotransferrin," Biochim Biophys Acta, **vol. 1820**, no. 3, pp. 218-25, Mar, 2012.

[2] *E. Sekyere, and D. R. Richardson*, "The membrane-bound transferrin homologue melanotransferrin: roles other than iron transport?," FEBS Lett, **vol. 483**, no. 1, pp. 11-6, Oct 13, 2000.

[3] *L. L. Dunn, E. O. Sekyere, Y. Suryo Rahmanto et al.*, "The function of melanotransferrin: a role in melanoma cell proliferation and tumorigenesis," Carcinogenesis, **vol. 27**, no. 11, pp. 2157-69, Nov, 2006.

[4] *Y. Suryo Rahmanto, L. L. Dunn, and D. R. Richardson*, "The melanoma tumor antigen, melanotransferrin (p97): a 25-year hallmark--from iron metabolism to tumorigenesis," Oncogene, **vol. 26**, no. 42, pp. 6113-24, Sep 13, 2007.

[5] *T. Takami, and I. Sakaida*, "Iron regulation by hepatocytes and free radicals," Journal of clinical biochemistry and nutrition, **vol. 48**, no. 2, pp. 103-106, 2011.

[6] *B. J. Cherayil*, "The role of iron in the immune response to bacterial infection," Immunologic research, **vol. 50**, no. 1, pp. 1-9, 2011.

[7] *N. Abbaspour, R. Hurrell, and R. Kelishadi*, "Review on iron and its importance for human health," Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences, **vol. 19**, no. 2, pp. 164-174, 2014.

[8] *A. Leibman, and P. Aisen*, "Distribution of iron between the binding sites of transferrin in serum: methods and results in normal human subjects," Blood, **vol. 53**, no. 6, pp. 1058-65, Jun, 1979.

[9] *P. Aisen, and I. Listowsky*, "Iron transport and storage proteins," Annu Rev Biochem, **vol. 49**, pp. 357-93, 1980.

[10] *C. A. Smith, B. F. Anderson, H. M. Baker et al.*, "Metal substitution in transferrins: the crystal structure of human copper-lactoferrin at 2.1-A resolution," Biochemistry, **vol. 31**, no. 18, pp. 4527-33, May 12, 1992.

[11] *A. Seidel, E. Bill, L. Häggström et al.*, "Complementary Mössbauer and EPR studies of iron(III) in diferric human serum transferrin with oxalate or bicarbonate as synergistic anions," Arch Biochem Biophys, **vol. 308**, no. 1, pp. 52-63, Jan, 1994.

[12] *M. Arruebo, N. Vilaboa, B. Sáez-Gutierrez et al.*, "Assessment of the evolution of cancer treatment therapies," Cancers, **vol. 3**, no. 3, pp. 3279-3330, 2011.

[13] *S. Tortorella, and T. C. Karagiannis*, "Transferrin receptor-mediated endocytosis: a useful target for cancer therapy," J Membr Biol, **vol. 247**, no. 4, pp. 291-307, Apr, 2014.

[14] *Z. M. Qian, H. Li, H. Sun et al.*, "Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway," Pharmacol Rev, **vol. 54**, no. 4, pp. 561-87, Dec, 2002.

[15] *T. R. Daniels, E. Bernabeu, J. A. Rodriguez et al.*, "The transferrin receptor and the targeted delivery of therapeutic agents against cancer," Biochim Biophys Acta, **vol. 1820**, no. 3, pp. 291-317, Mar, 2012.

[16] *T. R. Daniels, T. Delgado, G. Helguera et al.*, "The transferrin receptor part II: targeted delivery of therapeutic agents into cancer cells," Clin Immunol, **vol. 121**, no. 2, pp. 159-76, Nov, 2006.

[17] *X. Y. Sun, Q. Z. Gan, and J. M. Ouyang*, "Calcium oxalate toxicity in renal epithelial cells: the mediation of crystal size on cell death mode," Cell Death Discovery, **vol. 1**, pp. 15055, 11/23/online, 2015.

[18] *C. Miller, L. Kennington, R. Cooney et al.*, "Oxalate toxicity in renal epithelial cells: characteristics of apoptosis and necrosis," Toxicol Appl Pharmacol, **vol. 162**, no. 2, pp. 132-41, Jan 15, 2000.

[19] *M. T. Moryama, C. Domiki, K. Miyazawa et al.*, "Effects of oxalate exposure on Madin-Darby canine kidney cells in culture: renal prothrombin fragment-1 mRNA expression," Urol Res, **vol. 33**, no. 6, pp. 470-5, Dec, 2005.

[20] *B. C. Jeong, C. Kwak, K. S. Cho et al.*, "Apoptosis induced by oxalate in human renal tubular epithelial HK-2 cells," Urol Res, **vol. 33**, no. 2, pp. 87-92, May, 2005.

[21] *H. J. Han, M. J. Lim, and Y. J. Lee*, "Oxalate inhibits renal proximal tubule cell proliferation via oxidative stress, p38 MAPK/JNK, and cPLA2 signaling pathways," Am J Physiol Cell Physiol, vol. 287, no. 4, pp. C1058-66, Oct, 2004.

[22] *F. Kilár, and S. Hjertén*, "Fast and high resolution analysis of human serum transferrin by high performance isoelectric focusing in capillaries," Electrophoresis, **vol. 10**, no. 1, pp. 23-9, Jan, 1989.

[23] *T. Konecsni, and F. Kilár*, "Monitoring of the conjugation reaction between human serum transferrin and fluorescein isothiocyanate by capillary electrophoresis," J Chromatogr A, **vol. 1051**, no. 1-2, pp. 135-9, Oct 8, 2004.

[24] *J. H. Ward, J. P. Kushner, J. Kaplan,* "Regulation of HeLa cell transferrin receptors," J Biol Chem, **vol. 257**, no. 17, pp. 10317-10323-1982